Levodopa/Dopamine Analogs as Inhibitors of DNA Synthesis in Human Melanoma Cells

Michael M. Wick, M.D., Ph.D.
Dana Farber Cancer Institute, Department of Dermatology, Harvard Medical School, Boston, Massachusetts, U.S.A.

Compounds possessing the ortho-dihydroxybenzene moiety and structurally related to levodopa and dopamine appear to belong to a new class of biologically active agents that are capable of inhibiting DNA synthesis in a variety of normal and malignant eukaryotic cells. They appear to work in part through their properties as potent reducing agents, and thereby interfere with several redox reactions that are crucial to the synthesis of cellular DNA. Interestingly, the compounds can then be converted to their oxidized or quinone forms, which are powerful electrophiles, and further disrupt cellular metabolism. This latter mechanism appears to account for the relatively increased cytotoxicity observed in melanin-producing cells. In addition to providing potential new agents for the treatment of advanced malignant melanoma, these compounds might prove to be valuable tools in determining the factors that influence rates of DNA synthesis in normal and malignant cells. *J. Invest Dermatol 92:329S–331S, 1989*

Melanoma is a tumor whose increasing incidence, aggressive biologic behavior, and resistance to conventional antitumor agents have required the development of new approaches to chemotherapy. Melanoma cells, in the majority of cases, retain their ability to manufacture the pigment melanin, which is produced by the oxidation of amino-acid precursors. Specifically, tyrosine is converted to levodopa by tyrosinase which is uniquely found within melanocyte-derived cells. Levodopa is subsequently oxidized to melanin. Because this reaction occurs only within melanocytes, and melanoma cells generally contain much more tyrosinase than normal melanocytes, it might be possible to take advantage of this difference to design drugs that would be selectively toxic to melanoma cells. Potential agents would rely for their activity on the measurable phenotypic difference, melanin production, rather than on more-subtle growth differences between normal and malignant cells that conventional agents most often rely upon for selectivity. Furthermore, because the melanocyte serves a nonessential role in the host and cytotoxicity would be directed primarily against the melanocyte, it was envisioned that these drugs would be highly selective and relatively nontoxic. Most important, because these new agents have a novel mechanism of action, they would be ideal candidates for combination protocols with other conventional agents in the treatment of melanoma.

Clinical Studies In developing this approach, over 60 analogs of levodopa and dopamine have been prepared and evaluated. The most promising of these is 3,4-dihydroxybenzylamine (3,4-DHBA) (NSC 263475), which is currently undergoing evaluation at the National Cancer Institute prior to introduction to clinical trial. Pending the availability of new compounds, we were in a unique position of having both dopamine and levodopa available for study in clinical settings, although they were less effective in experimental systems. Levodopa, in particular, was an attractive candidate because of its ability to cross the blood brain barrier, and CNS metastases involving melanoma continue to be a difficult therapeutic problem. Dopamine had the advantage of being available for parenteral administration.

Because there is currently no effective therapy for the treatment of advanced melanoma in humans, we sought to determine if cytotoxic levels could be achieved using dopamine, and if biochemical inhibitory effects on tumor cells in vivo as measured by thymidine incorporation, could be demonstrated. This is a prerequisite for a subsequent demonstration of an antitumor response [1].

Four patients with histologically proven metastatic malignant melanoma for whom no other treatment was available were accepted into the study. Patients were premedicated with propranolol, and dopamine infusions were maintained at maximally tolerable levels for 48 to 120 h, with constant monitoring of cardiovascular parameters. Bone-marrow aspirates were taken immediately prior to and 24 h after the initiation of therapy and processed for labeling and scintillation indices. Each patient had multiple cutaneous nodules, which permitted excisional biopsy of the tumor and determination of labeling index in the biopsy specimen before and 24 h after infusion was completed. Plasma levels were determined to assess whether or not they were in the tumoricidal range.

Labeling and scintillation indices of the tumors were determined by a modification of reported techniques using single-cell suspensions prepared from tumor biopsies. The major toxicity was related to the cardiovascular effects of dopamine. At the maximally tolerated dose of 20 μg/kg per min, steady-state plasma concentrations between 10^{-5} and 10^{-6} M were achieved. Comparison of the labeling index of the tumor, biopsied prior to the start of treatment and again 1 d after cessation of treatment, indicated a consistent tenfold reduction in labeling index. Bone-marrow samples processed before and 24 h after the start of infusion consistently showed a decrease in the incorporation of radioactively labeled nucleotide precursors, but no change in labeling index.

The combination of levodopa/carbidopa was then used in an attempt to circumvent the toxicities of dopamine as well as to deliver the drug to the CNS [2]. Of 12 patients treated to date, eight are evaluable, and there have been four significant clinical responses. Significantly, the plasma levels achievable with levodopa were in tumoricidal range, as predicted by *in vitro* assays (10^{-5} M). One patient had a complete resolution of a CNS lesion, as measured by a CAT scan, and a corresponding improvement in symptoms.

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Reprint requests to: Michael M. Wick, M.D., Ph.D., Dana Farber Cancer Institute, 44 Binney Street, Boston, MA 02115

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Pending the availability of improved analogs, further study of the use of levodopa/carbidopa as therapy for malignant melanoma in humans appears warranted.

With the advent of dopamine analogs that are devoid of cardiovascular effects, the new drugs should be able to achieve the requisite concentrations in plasma without this dose limiting toxicity. Significantly, 3,4-dihydroxybenzylamine shows enhanced therapeutic activity in all experimental models, primarily through an increase in the maximally tolerated dose.

Mechanistic Studies Our recent efforts have focused upon the mechanistic processes underlying the remarkable selectivity that is exhibited by what are essentially members of a new class of antiproliferative agents characterized by the possession of the ortho-dihydroxybenzene moiety. These efforts were preceded by studies that examined the effects of levodopa and closely related ortho-dihydroxybenzene derivatives on pigmented and nonpigmented clones of human melanoma cells growing in tissue culture. Levodopa itself has very little effect on cells that do not form pigment, but it appears to be highly inhibitory to the growth of pigment-producing cells. We extended these results to *in vivo* systems using the B-16 melanoma in mice, as well as human melanomas transplanted into nude mice. We observed significant and reproducible antitumor activity in each of these systems, with a significant prolongation of survival of treated mice as compared with nontreated controls [3,4].

In a typical experiment where mice bearing the B-16 melanoma were treated daily, the treated mice lived up to 50% longer than their nontreated counterparts. In general, as far as can be predicted from preclinical studies, these results suggest that these antitumor drugs, although novel in structure, are at least as effective as conventional antitumor drugs tested in these models. In addition, as a novel approach to chemotherapy, these derivatives offer unique opportunities to utilize both conventional and new approaches to combination chemotherapy. Thus, two groups have already begun to explore ways to enhance the antitumor effects of these compounds. One approach by a group at Merrell Dow utilized difluoromethylornithine (which may lead to the induction of tyrosinase activity) in combination with 3,4-dihydroxybenzylamine. Their results showed that this combination resulted in a significant increase in survival time of mice bearing the B16 melanoma [5]. A different approach by Meadow et al [6,7] utilizes alterations in diet, i.e., restricting levels of tyrosine and phenylalanine and/or addition of sodium ascorbate. They demonstrated that the antitumor effectiveness of levodopa derivatives can be increased by controlling the animal's diet.

Once the basic antitumor effects were established *in vitro* and *in vivo,* we began to focus the mechanistic studies upon explaining the characteristic biochemical effects of the poly-hydroxybenzylamine compounds. Added impetus for the development of this line of investigation was given by the observation that the dihydroxybenzene derivatives were able to inhibit DNA synthesis in a variety of non-melanocyte-derived eukaryotic and prokaryotic cells, including leukemia cells, squamous cell carcinoma, mushrooms, and bacteria. Our research in this area has led to an explanation of this general phenomenon that describes the ways in which the inhibitory effects of the ortho-dihydroxybenzene compounds may differ between pigmented melanoma cells and nonpigmented cells. We have discovered a possible difference in the inhibitory mechanism of action between various derivatives in different cell types, and we will review some of this evidence.

The initial investigations with these drugs revealed that they inhibited thymidine incorporation *in vitro* while having a minimal effect on RNA or protein synthesis [8]. The time course for the effect of 3,4-DHBA and dopamine upon thymidine incorporation indicated a rapid suppression of DNA synthesis (ca. 15 min following exposure), which suggests the inhibition of a key reaction in DNA replication. The effect of these compounds on cell-cycle progression was analyzed by means of sequential DNA histograms using flow cytofluorometry. The results demonstrate that the cells are arrested at the G_1/S interface, suggesting a block in the metabolism of a precursor for DNA synthesis that prevents cells from entering the S phase. Finally, the survival curves from the clonogenic assays are similar to drugs that are known inhibitors of DNA synthesis. These *in vitro* findings suggested that the immediate cellular effect of these catechols is a direct and selective inhibition of DNA synthesis.

Inhibitors of DNA synthesis can be assigned to one of two classes: a) those that inhibit the supply of deoxyribonucleotides or b) those that directly interfere with the polymerase reaction, either by inhibiting the enzyme DNA polymerase or by altering the structure of the initiator-template. Using an isolated enzyme system as a model of a pigmented melanoma cell, we have been able to demonstrate that the dihydroxy derivatives of levodopa in the presence of the polyphenol oxidase, tyrosinase (which catalyzes the oxidation of tyrosine to melanin in melanocytes) are capable of inhibiting sulfhydryl-dependent DNA polymerases [9]. In this same study, we also described evidence for the molecular mechanism of action for tyrosinase activated 3,4-DHBA and obtained further evidence using preincubation, dialysis, and kinetic analyses, which confirmed that the inhibition of replication by oxidized 3,4-DHBA occurs at the level of DNA polymerase. This observation was extended by a Hill plot analysis which showed that there are two inhibitor sites involved in the inactivation of the enzyme. Second, the inhibition of polymerase alpha by tyrosinase activated 3,4-DHBA is irreversible and cannot be restored by removal of the drug and addition of a reducing agent, e.g., dithioerythritol (DTE). This result, along with the demonstration that 3,4-DHBA titrates the enzyme by reacting stoichiometrically, suggests that the inactivation involves the formation of a covalent bond, possible via a Michael addition of SH groups to the semiquinone species 3,4-DHBA. While earlier work suggested that inactivation of polymerase by quinols may be mediated by superoxide and hydroxyl-free radicals, it now appears more likely that inactivation is the result of direct titration of the enzyme by the drug and not a simple, indirect oxidation. Last, it was shown that DNA protects the enzyme, suggesting that the site of inhibition is at or near the active site [9].

The enzymes involved in the *de novo* production of DNA substrates, deoxyribonucleotides, include several kinases, enzymes involved in thymidylate synthesis, and ribonucleotide reductase. Ribonucleotide reductase is considered to be the key enzyme for synthesis of dNTP, because it catalyzes not only a rate limiting step, but also the first step unique to DNA synthesis. This enzyme catalyzes the one-step direct replacement of the 2′-OH group of ribonucleotide diphosphate with hydrogen. This reduction is accomplished by a radical dithiol system where the radical may involve a tyrosine residue with an unpaired electron at the hydroxyl group. Ribonucleotide reductase was a logical choice to pursue as a possible alternative or additional site of action for the ortho derivatives. Support for ribonucleotide reductase as a site of action of 3,4-DHBA has been presented by Elford et al [10], who demonstrated that reduced polyhydroxybenzene derivatives, including five levodopa analogs which we had been investigating, are potent inhibitors of isolated ribonucleotide reductase. We have been able to confirm and extend Elford's results using partially purified ribonucleotide reductase [11]. Our results showed that the inhibition of ribonucleotide reductase by 3,4-DHBA was reversible and competitive with the reducing substrate, DTE, i.e., the inhibitory activity was abolished at high DTE concentrations. 3,4-DHBA was not competitive toward the nucleotide diphosphate substrate. Hydroxyurea (HU), a known inhibitor of ribonucleotide reductase, has a partially competitive relationship with the dithiol substrate, which suggests that these drugs do not inhibit ribonucleotide reductase by the same mechanism. To investigate this possibility, we examined the type of inhibitory interaction (synergistic, additive, or antagonistic) which results from combining these two inhibitors. An isobologram analysis of the combined inhibitory effects of hydroxyurea and 3,4-DHBA on ribonucleotide reductase suggested a synergistic interaction [11]. This result agrees with the suggestion that 3,4-DHBA does not act at the same site as HU.

The inhibitory effect of several ortho-dihydroxybenzene deriva-

tives on ribonucleotide reductase and DNA polymerase activity was examined using permeabilized cells. We were able to show that preincubation of cells with the drugs did not lead to increased inhibition of ribonucleotide reductase, indicating that the inhibitory effect of the ortho-dihydroxybenzene derivatives was immediate. On the other hand, there was no immediate effect on DNA polymerase activity by these levodopa analogs. Inhibition of DNA polymerase was observed only after 1 h preincubation with the drugs [11].

To describe further the action of these novel antitumor agents, we examined the action of these agents upon a third enzyme that is crucial to the control of DNA synthesis, thymidylate synthase. The *in vitro* analysis of the effect of inhibitors of the various enzymes involved in DNA synthesis is made difficult because the cell membrane is impermeable to their immediate substrates. The use of permeabilized cells is one technique that we have used to circumvent this problem. An alternative *in situ* approach for the study of the enzyme thymidylate synthase has been developed which utilizes charcoal filtration to isolate [^{3}H]-H_2O released from [5-^{3}H] deoxyuridylate. The initial studies were designed to determine the effects of the antitumor agents bearing the dihydroxybenzene moiety upon thymidylate synthase activity *in situ* [12,13]. Significant inhibition of thymidylate synthase was observed with each of the ortho-dihydroxybenzene analogs with 3,4-DHBA showing the greatest inhibitory activity (IC_{50} 176 μM). We examined the inhibitory effect of 3,4-DHBA on the enzyme's activity using a partially purified cell-free extract to determine if the observed inhibitory effect upon thymidylate synthase was directly or indirectly mediated. Similar to the findings of Reddy and Pardee [14], who examined the effects of the ribonucleotide reductase inhibitor, hydroxyurea, on thymidylate synthase, we also found that while the catechols had an inhibitory effect on thymidylate synthase *in situ,* they had no inhibitory effect against the isolated enzyme. This result suggests that the *in situ* inhibitory effect involves an indirect mechanism. One possible indirect mechanism is that the quinol must be activated, oxidized to the quinone form, before it is capable of inhibiting thymidylate synthase. We were able to show that the oxidized form of 3,4-DHBA is a very potent irreversible inhibitor of this enzyme ($IC_{50} < 100$ μM). However, in the nonpigmented cell lines used in the thymidylate synthase studies, the *in situ* inhibition of this enzyme was found to be reversible. Another possible indirect mechanism invokes the concept of the replitase, the putative multienzyme complex for the synthesis of DNA [14]. Thus, a drug may directly affect one enzyme of this complex (e.g., 3,4-DHBA inhibits ribonucleotide reductase) which leads, as the result of allosteric interactions, to the indirect inhibition of other enzyme(s) of the replitase, e.g., thymidylate synthase.

The dihydroxybenzene derivatives related to levodopa and dopamine have been shown to inhibit three enzymes related to DNA synthesis ribonucleotide reductase, DNA polymerase, and thymidylate synthase. From our results using the permeabilized cell technique and from our *in situ* assays, it would appear that the most likely initial event is the inhibition of ribonucleotide reductase. If the inhibition of ribonucleotide reductase is sufficient, perhaps resulting in a reduction in deoxynucleotide pools below a critical threshold level, then the inhibition of DNA synthesis is immediate. Our results appear to indicate that 3,4-DHBA need not inhibit ribonucleotide reductase completely in order to totally suppress DNA synthesis. At lower concentrations of 3,4-DHBA, the inhibitory effect on DNA synthesis is delayed (ca. 15 min at 0.2 mM), possibly until the deoxynucleotide pools are sufficiently reduced. In contrast, the inhibition of thymidylate synthase is delayed regardless of the drug concentration for about 20 min. The inhibition of DNA polymerase is also delayed at each of the drug concentrations [11]. The time of onset of inhibition of these two enzymes does not correlate with the time of onset of inhibition of DNA synthesis, whereas the time of onset of inhibition of ribonucleotide reductase either coincides or precedes the inhibition of DNA synthesis. One possible explanation for the delayed inhibitory effect on DNA polymerase and thymidylate synthase is that the drug must be activated to the quinone in order to inhibit these enzymes. We have previously shown that the oxidized form is a very potent inhibitor of DNA polymerase [9] and that the quinone is also a very potent inhibitor of thymidylate synthase [12]. In cells of high oxidative potential, e.g., melanoma, this quinone mediated mechanism might be uniquely important.

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